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# Environmental influences on prolactin cell development in the cyprinodont fish, *Cynolebias whitei*

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**Summary.** A combined immunocytochemical and morphometric study on the development of the prolactin (PRL) cells of the annual cyprinodont *Cynolebias whitei*, transferred as newly hatched larvae to water with different salinities and/or  $\text{Ca}^{2+}$ -concentrations, was carried out. The percentage of the pituitary volume occupied by PRL cells and the affinity of PRL cells for immunocytochemical staining were used as criteria for their activity. Exposure of the larvae for one day to salt water (260 mOsm/kg) led to a significant reduction in the pituitary volume occupied by PRL cells, indicating an osmoregulatory function of PRL shortly after hatching. In fish reared in diluted artificial seawater (70 and 260 mOsm/kg) or  $\text{Na}^+$ -enriched fresh water the development of PRL cells was significantly retarded, but such an effect was not observed in fish placed in  $\text{Ca}^{2+}$ -enriched fresh water. These experiments show that in *C. whitei* the development and activity of PRL cells are influenced by changes in environmental osmolarity and not by changes in ambient  $\text{Ca}^{2+}$ -concentration.

**Key words:** Prolactin cells – Ontogenetic development – Pituitary – Teleost fish – Calcium ions

The importance of the pituitary hormone prolactin (PRL) for osmoregulation in adult fishes is well established (for reviews: Schreibman et al. 1973; Loretz and Bern 1982). However, the morphology and function of PRL cells in young fish has been described in only a limited number of reports and the timing of the differentiation of the PRL cells in the developing pituitary is largely unknown (for review: Schoots et al. 1983). Immunocytochemical evidence for the presence of PRL cells in the pituitary of two cyprinodont fishes at the time of hatching has been published recently (Schoots et al. 1983). Ichikawa (1973) and Leatherland and Lin (1976) have exposed newly hatched fish to environments with different salinities to investigate the osmoregulation of these animals. The differences that appear in the size of PRL cells or PRL-cell nuclei suggest that PRL controls osmoregulation shortly after hatching. Long-term effects of environmental salinity on the activity of PRL cells in developing fish have been reported for two viviparous cyprinodonts (Holtzman et al. 1972; Ball and Ingleton 1973).

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Light- and electron-microscopical studies of PRL cells of adult fish show a high activity in fresh-water-adapted (FW) fish but much lower activities in fish from seawater (SW) or diluted seawater. There are conflicting observations concerning the influences of osmolarity and concentrations of  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -ions on the activity of PRL cells. In the stickleback, *Gasterosteus aculeatus*, (Wendelaar Bonga 1978a, b), in tilapia, *Sarotherodon mossambicus* (Wendelaar Bonga et al. 1983) and in the eel, *Anguilla anguilla*, (Oliverieu and Oliverieu 1983) PRL cells are inactivated in  $\text{Ca}^{2+}$ -enriched FW. In the goldfish, *Carassius auratus*, (Oliverieu et al. 1982a) and *Gambusia* sp. (Dubourg et al. 1983), the addition of  $\text{CaCl}_2$  to deionized water does not affect PRL cells. Exposure of fish to Ca-free (diluted) SW results in the slight activation of PRL cells in *C. auratus* (Oliverieu et al. 1983) and *A. anguilla* (Oliverieu and Oliverieu 1983). PRL cells of *G. aculeatus* are activated when  $\text{Ca}^{2+}$  (Ogasawara and Yamada 1979) or  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Wendelaar Bonga 1978a, b) are omitted from SW. The removal of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  is also necessary to activate PRL cells in *S. mossambicus* (Wendelaar Bonga et al. 1981). PRL cells of *Fundulus heteroclitus* are only marginally activated when fish are reared in  $\text{Ca}^{2+}$ -deficient SW (Ball et al. 1982). Thus, the effects of the  $\text{Ca}^{2+}$ -concentration on the activity of PRL cells are not uniform and are possibly species-specific.

The aim of the present investigation is to determine the activity of PRL cells shortly after hatching and to study the influence of environmental osmolarity and calcium concentration on the development of these cells in the annual cyprinodont *Cynolebias whitei* (Myers 1942) using a combined immunocytochemical and morphometric approach.

## Materials and methods

### Experimental animals

Eggs of the annual fish *Cynolebias whitei* were stored in moist peat for 3 months after spawning by which time they had developed to the prehatching diapause stage (stage 43, according to Wourms 1972). Hatching was induced by immersion of the peat in unaerated fresh water (FW). The larvae hatched after approximately 6 h. Shortly after hatching the following two experiments were carried out:

1. The animals were transferred to FW with seasalt (Wiegant GmbH & Co) added to give an osmolality of 70 and 260 mOsm/kg. The salt concentrations were increased daily to reach the final concentration 48 h after hatching. Groups of 10 animals were fixed after 1 day and



after 1, 2, 3 and 5 weeks. Fish of the same ages, reared in FW (6 mOsm/kg) and larvae fixed just before and 18 h after hatching were used as controls.

2. Fish were exposed to:

a) Fresh water (FW; 6 mOsm/kg; 2 mM  $\text{Na}^+$ ; 0.9 mM  $\text{Ca}^{2+}$ )

b) Diluted artificial seawater (1/3 SW; 260 mOsm/kg; 130 mM  $\text{Na}^+$ ; 3.5 mM  $\text{Ca}^{2+}$ )

c)  $\text{Ca}^{2+}$ -enriched FW (11 mOsm/kg; 2 mM  $\text{Na}^+$ ; 3.5 mM  $\text{Ca}^{2+}$ )

d)  $\text{Na}^+$ -enriched FW (260 mOsm/kg; 130 mM  $\text{Na}^+$ ; 0.8 mM  $\text{Ca}^{2+}$ )

e)  $\text{Ca}^{2+}$ - and  $\text{Na}^+$ -enriched FW (260 mOsm/kg; 130 mM  $\text{Na}^+$ ; 3.5 mM  $\text{Ca}^{2+}$ ).

The salt concentrations were increased twice a day by 25% of the final concentrations. The experiment started 2 days after hatching. All animals were fixed after 3 weeks of exposure.

#### *Histological and immunocytochemical procedures*

All fish were anaesthetized in 0.3% MS222 (Sandoz) and fixed for 24, 48 or 72 h (depending on their size) in a mixture of glutaraldehyde (25%, aqueous solution) and picric acid (saturated aqueous solution) (ratio 1:3) with 1% acetic acid (GPA) at 4°C. Following dehydration, the tissue was embedded in paraplast and the whole pituitary was serially sectioned at a thickness of 4 µm in the sagittal plane. Six to twelve sections, at equal intervals, were stained with:

a) Cleveland and Wolfe's trichrome stain. Staining times were: 3 min in Erythrosine (1% in distilled water (DW)), 4.5 min in Orange G (2.1% in 1% phosphotungstic acid in DW) and 1.5 min in Aniline Blue (1% in DW with 1% 1N HCl) followed by rapid dehydration in absolute ethanol and clearing in xylene.

b) Immunoperoxidase staining according to Sternberger (1979) using a rabbit antiserum to ovine PRL (obtained from Dr. J. Matthey, Agricultural University, Wageningen). Following pretreatment with 0.3%  $\text{H}_2\text{O}_2$  in absolute methanol (to inhibit endogenous peroxidase activity, 30 min) and normal goat serum (1:5 dilution in phosphate-buffered saline, PBS, 30 min), the sections were incubated for 18 h at 4°C with 1:400 diluted anti-PRL serum. On the following day, the sections were incubated at room temperature with goat-anti-rabbit serum (Gar IgG (Fc), Nordic, 1:30, 2 h), PAP complex (rabbit PAP, Dakopatts, 1:90, 1 h) and 0.075% DAB (Sigma) in 0.01%  $\text{H}_2\text{O}_2$  in Tris buffer (15 min). All sera were diluted in PBS.

Specificity of the immunocytochemical (ICC) staining was confirmed by omitting the various steps one by one and by replacing the antiserum with normal rabbit serum or with antiserum absorbed with various amounts of ovine PRL. These controls were carried out with sagittal sections of the pituitary of GPA-fixed fishes at 3 months of age. From these controls, it was concluded that the ICC procedure results in a specific staining of the prolactin cells in the pituitary.

#### *Morphometric procedure*

The percentage of the pituitary volume that is occupied by PRL cells (% PRL) was measured by means of point-counting volumetry on drawings of sections, selected at regular distances from the whole pituitary, at a final magnifica-

tion of  $1334\times$  for small, to  $127\times$  for large pituitaries. Cleveland and Wolfe's trichrome or immunocytochemically-stained sections were used. The volume of the whole pituitary gland and the volume occupied by PRL cells were subsequently calculated from the point-counting data using the method of Weibel (1979). Each fish was measured separately. The results were statistically analysed using the U-test of Wilcoxon, Mann and Whitney (Sachs 1968).

#### **Results**

The general morphology of the pituitary gland of *C. whitei*, as revealed by histological staining, is similar to that of other cyprinodonts (for review: Holmes and Ball 1974). PRL cells can be easily identified in control fish from normal fresh water. Following Cleveland and Wolfe's trichrome stain the PRL cells stain red whereas following the immunocytochemical procedure they stain intensely brown.

In larvae and young fish up to three weeks of age, PRL cells are found solely in the rostral pars distalis (RPD), which is non-follicular in cyprinodonts (Figs. 1, 2). In five-week-old fish, besides PRL cells in the RPD, several isolated cell groups with the staining properties of PRL cells are found in the proximal pars distalis (PPD) and the pars intermedia (PI) (Fig. 4). In older fish, the number of isolated PRL cell groups increases with age.

#### *Experiment 1*

In all FW fish and in fish exposed to 260 mOsm/kg for one day, PRL cells stain intensely following the ICC procedure (Figs. 1, 2, 4). The percentage of pituitary volume occupied by PRL cells (% PRL) increases slightly from  $17.2\pm 0.8\%$  in prehatching larvae to  $21.2\pm 1.8\%$  in fish of five weeks of age (Fig. 6).

In fish from water with an osmolality of 70 mOsm/kg, the affinity of PRL cells for ICC staining is reduced. Only a very weak affinity to the antiserum is observed in fish exposed to 260 mOsm/kg for one week or longer (Figs. 3, 5). In Cleveland and Wolfe's trichrome-stained-sections PRL cells remain identifiable as red cells.

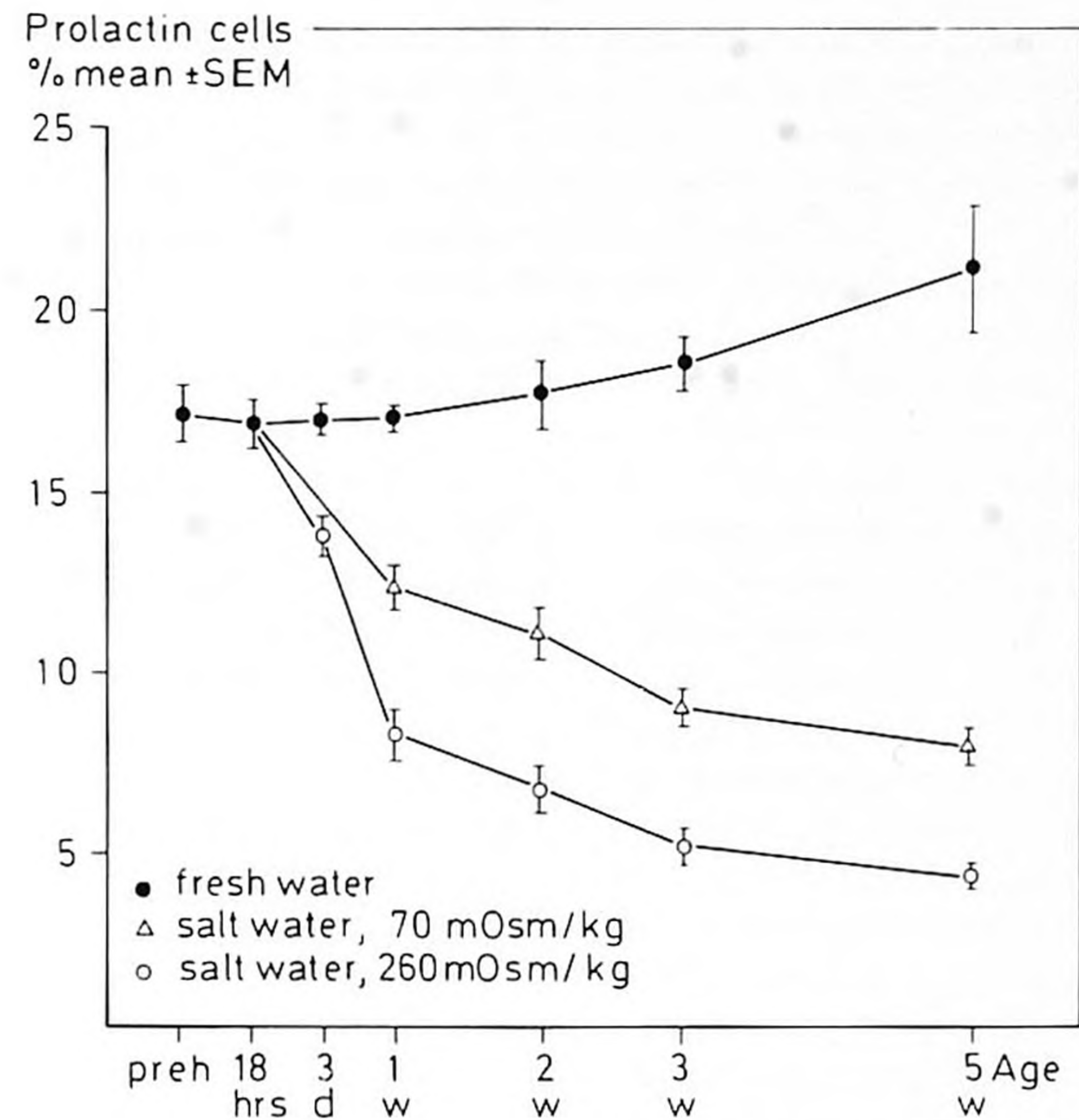
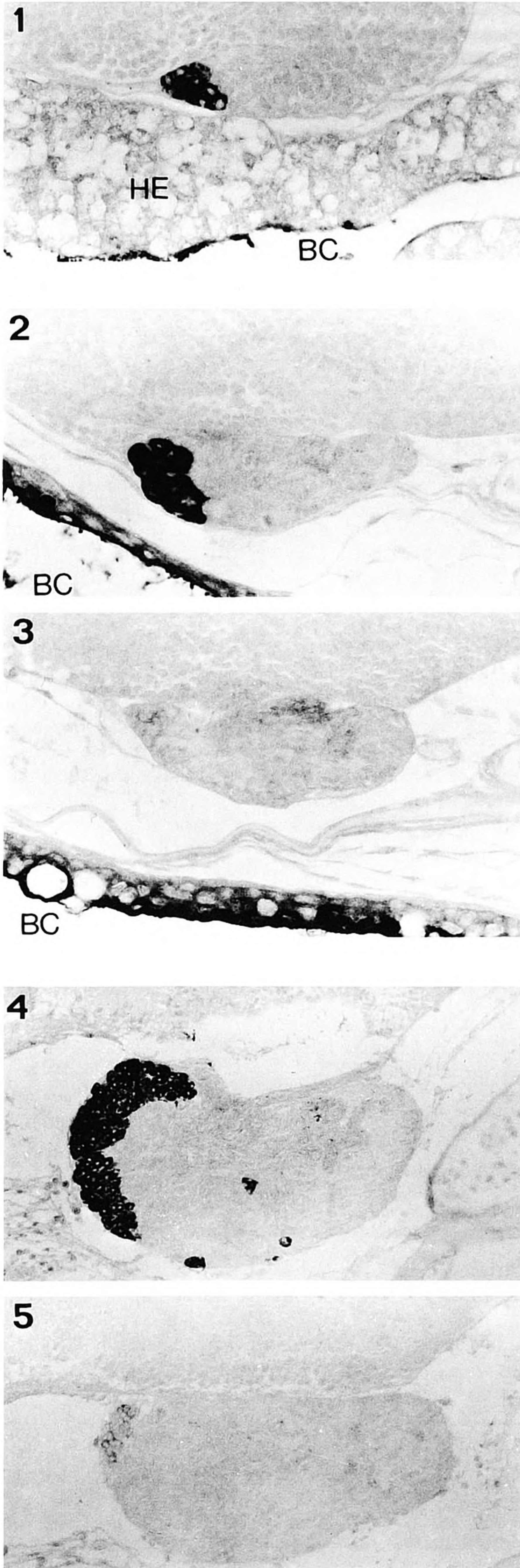
In both salinities, the %PRL decreases strongly with age. After one day in 260 mOsm/kg, the %PRL is already significantly decreased (from  $17.0\pm 0.4$  in FW to  $13.8\pm 0.6\%$  in 260 mOsm/kg;  $P < 0.01$ ). This percentage decreases further until it stabilises after about three weeks at approximately 8% in fish in 70 mOsm/kg and approximately 4% in fish in 260 mOsm/kg (Fig. 6).

In spite of differences in the size of the pituitary of fish at two and three weeks old, the total pituitary volume at five weeks of age is approximately the same in all salinities (Table 1). The lower %PRL in fish developing in salt water at all ages is reflected in the lower volume occupied by PRL cells in these fish when compared with FW fish (Table 1). These lower volumes are a result of the reduced number of PRL cells in salt-water-adapted fish (compare Figs. 4 and 5).

#### *Experiment 2*

In  $\text{Ca}^{2+}$ -enriched FW, the affinity of PRL cells to ICC staining is the same as in FW. In 1/3 SW, in  $\text{Na}^+$ -enriched FW and in  $\text{Na}^+$ - and  $\text{Ca}^{2+}$ -enriched FW, no immune reaction is observed.





**Fig. 6.** Development of the part of the pituitary occupied by prolactin cells in fish reared in fresh water (—●—) and salt water (70 mOsm/kg (—△—) and 260 mOsm/kg (—○—)). Results of point-counting volumetry of Cleveland and Wolfe's trichrome or immunocytochemically stained sections. Mean percentage  $\pm$  SEM for each group; see Table 1 for the number of fish/group

The part of the pituitary occupied by PRL cells is large in FW and  $\text{Ca}^{2+}$ -enriched FW ( $20.0 \pm 0.8\%$  and  $16.9 \pm 1.3\%$  resp.;  $0.05 < p < 0.1$ ). In the other media this parameter is as low as in 1/3 SW ( $4.7 \pm 0.4\%$ ; Fig. 7).

## Discussion

### Identification and localization of PRL cells

Although not all antisera raised against purified mammalian or teleostean prolactins cross-react with PRL cells of all the species of teleosts investigated (Rawdon 1979; Nagahama et al. 1981; Naito et al. 1983), ICC procedures with anti-ovine PRL serum have been successfully used to identify PRL cells in many teleosts (for review: Follénus et al. 1978; Nagahama et al. 1981). Verification of the results of ICC staining with histological staining and physiological data is, however, necessary.

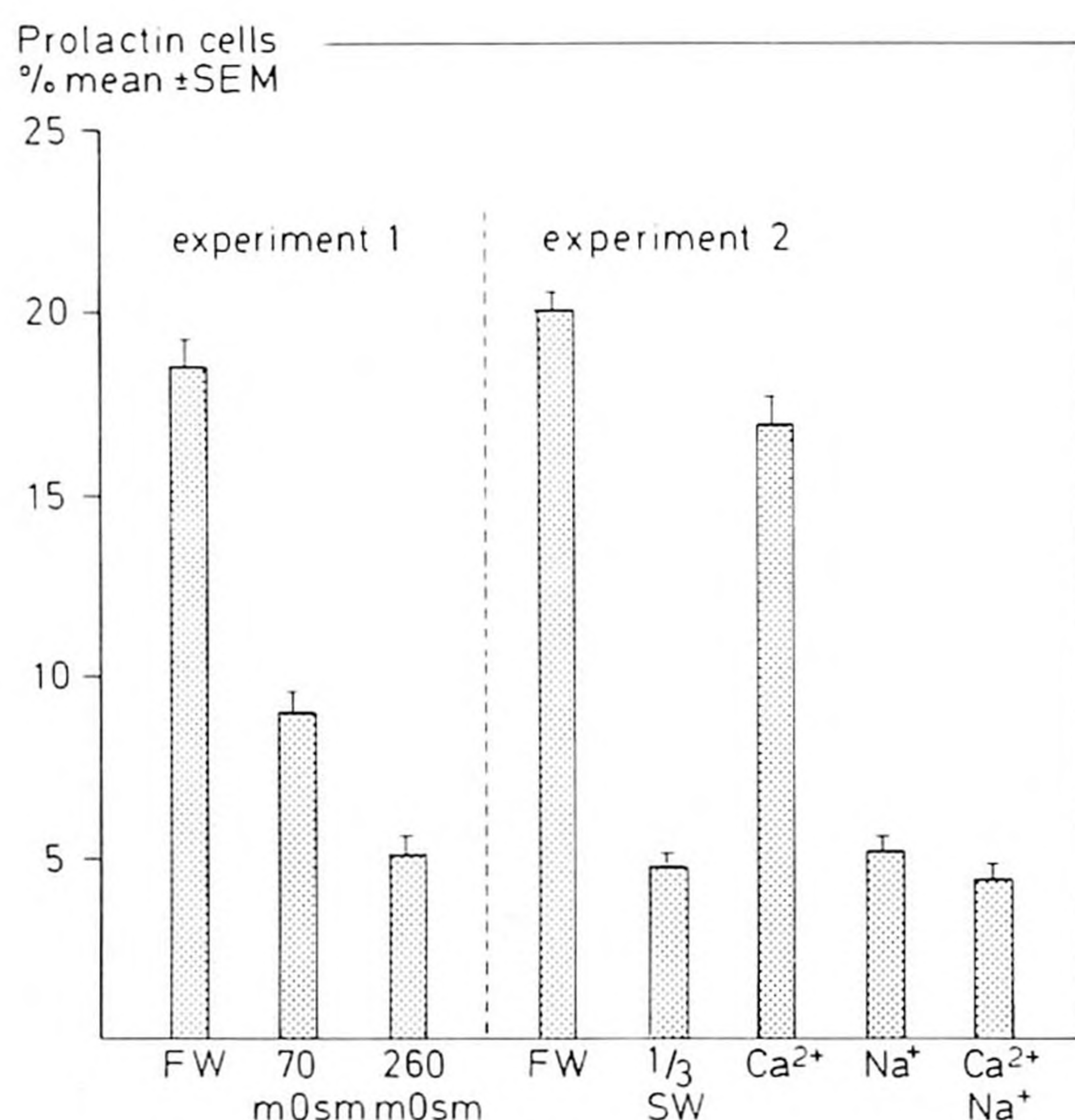
We have identified PRL cells of *Cynolebias whitei* using Cleveland and Wolfe's trichrome and immunocytochemical staining procedures. PRL cells stain red with Cleveland and Wolfe's trichrome and show a very strong affinity to anti-ovine PRL serum in the ICC procedure. The morphometric variations of these cells in fish adapted to fresh water or salt water reflect their involvement in osmoregulation.

**Figs. 1–5.** Mid-sagittal sections through the pituitary of *Cynolebias whitei*, stained immunocytochemically with an antiserum to ovine prolactin. The rostral pars distalis shows immunoreactive prolactin cells in pre-hatching larvae (**Fig. 1**:  $\times 400$ ) and fish reared in fresh water (**Fig. 2**: 1 week,  $\times 400$ ; **Fig. 4**: 5 weeks,  $\times 150$ ); in fish from salt water (260 mOsm/kg) immunostaining is almost absent (**Fig. 3**: 1 week,  $\times 400$ ; **Fig. 5**: 5 weeks,  $\times 190$ ). The staining in the buccal epithelium in 1-week-old fish also occurs when the antiserum is replaced by normal rabbit serum. HE hatching enzyme cells; BC buccal cavity



**Table 1.** Pituitary volumes and volumes occupied by prolactin cells in fish reared in fresh water and salt water (70 and 260 mOsm/kg); means  $\pm$  SEM ( $\times 10^6 \mu\text{m}^3$ )

Age	Pituitary volume	Pituitary volume occupied by PRL cells	Number of fish
Fresh water			
prehatching	$0.060 \pm 0.003$	$0.010 \pm 0.001$	11
18 hours	$0.056 \pm 0.002$	$0.009 \pm 0.0006$	13
3 days	$0.070 \pm 0.003$	$0.012 \pm 0.0006$	6
1 week	$0.161 \pm 0.011$	$0.027 \pm 0.002$	25
2 weeks	$0.357 \pm 0.026$	$0.063 \pm 0.007$	5
3 weeks	$0.677 \pm 0.071$	$0.126 \pm 0.014$	5
5 weeks	$4.89 \pm 0.52$	$1.01 \pm 0.08$	6
Salt water			
70 mOsm/kg			
1 week	$0.154 \pm 0.010$	$0.019 \pm 0.001$	9
2 weeks	$0.463 \pm 0.034$	$0.052 \pm 0.005$	9
3 weeks	$0.643 \pm 0.085$	$0.057 \pm 0.008$	9
5 weeks	$4.32 \pm 0.54$	$0.339 \pm 0.047$	7
260 mOsm/kg			
3 days	$0.075 \pm 0.005$	$0.011 \pm 0.0001$	5
1 week	$0.217 \pm 0.024$	$0.017 \pm 0.001$	8
2 weeks	$0.801 \pm 0.131$	$0.051 \pm 0.007$	7
3 weeks	$1.85 \pm 0.26$	$0.090 \pm 0.008$	8
5 weeks	$5.42 \pm 0.90$	$0.222 \pm 0.029$	7

**Fig. 7.** The part of the pituitary occupied by prolactin cells (mean percentage  $\pm$  SEM) in three-week-old fish. Experiment 1: fish reared in fresh water (FW) and salt water (70 mOsm/kg and 260 mOsm/kg). Experiment 2: fish reared in FW, diluted artificial seawater (1/3 SW), Ca<sup>2+</sup>-enriched FW (Ca<sup>2+</sup>), Na<sup>+</sup>-enriched FW (Na<sup>+</sup>) and Ca<sup>2+</sup>- and Na<sup>+</sup>-enriched FW (Ca<sup>2+</sup> Na<sup>+</sup>)

In animals up to three weeks of age, PRL cells form a compact cell mass in the RPD. Small groups of cells with the staining properties of PRL cells appear dispersed in the PPD and the PI in animals of five weeks old; on the basis of their immunoreactivity we conclude that they are also PRL cells. These cells are strongly erythrosinophilic and therefore not homologous to the PAS-positive PRL

immunoreactive cells observed by Rawdon (1979) in *S. mossambicus*. Small groups of PRL cells have also been observed in the PI of mature chum salmon (*Oncorhynchus keta*) and rainbow trout (*Salmo gairdneri*) by Naito et al. (1983). These authors have suggested that PRL cells may have migrated to the PI during development of the pituitary. Such a migration may also have caused the dispersed location of PRL cells in *C. whitei*. If so, this migration must have started between three and five weeks of age.

In the present study, we have used the development of PRL cells, expressed as a percentage of the pituitary volume occupied by PRL cells and the affinity of PRL cells to the ICC procedure, as criteria to study the influence of ambient salinity and Ca<sup>2+</sup>-ion concentration on the activity of PRL cells in *C. whitei*.

#### Development of PRL cells in various ambient salinities

The affinity of PRL cells for ICC staining is decreased in fish acclimatized to water with an osmolality of 70 or 260 mOsm/kg when compared with PRL cells of fish reared in FW. This decreased affinity indicates that the amount of PRL stored in PRL cells of fish reared in salt water is smaller than in fish from fresh water. This is in agreement with previously published data. Utida et al. (1971) have concluded from the effects of injected pituitary homogenates on plasma sodium concentration that pituitaries of FW-adapted medaka (*Oryzias latipes*) contain more PRL than pituitaries of SW-adapted fish. Ball and Ingleton (1973) have reported similar results for *Poecilia latipinna*, using a combined electrophoretic and densitometric method to determine the amount of PRL in pituitary homogenates. Measurement of the amount of PRL in the pituitary of *S. mossambicus* adapted to FW or artificial seawater with a homologous radioimmunoassay shows a decreased PRL content in the pituitaries of sea water-adapted fish (Nicoll et al. 1981).

During development, a progressive decrease of the %PRL is observed in fish reared in salt water. This decrease is a result of a reduced growth rate of PRL cells in fish developing in salt water when compared with fish from fresh water. Ball and Ingleton (1973) have reported similar findings for *P. latipinna* reared in different salinities during the two months from birth.

We conclude from our data that the degree of development of the PRL cells is closely related to the need for PRL for the maintenance of water and ion homeostasis in certain environments.

#### PRL function in young fish

The strong affinity for anti-PRL antiserum in PRL cells in prehatching larvae indicates a considerable storage of PRL in these cells. We have recently reported evidence that PRL may be involved in the endocrine regulation of hatching (Schoots et al. 1983).

The decrease of the %PRL after only one day of exposure to 260 mOsm/kg, when compared with FW controls, demonstrates that PRL cells in these young fish respond to salt water. This indicates that the osmoregulatory function of PRL cells is developed shortly after hatching. The inactivation of PRL cells in *Oncorhynchus kisutch* alevins transferred to 65% SW (Leatherland and Lin 1976) and the increase in the size of PRL cell nuclei in larvae of the



marine teleost *Hexagrammus atakii* transferred to FW (Ichikawa 1973), have also led to the conclusion that PRL cells are involved in the control of osmoregulation shortly after hatching.

#### *Effects of $\text{Ca}^{2+}$ on the PRL cell development in *C. whitei**

In the second experiment, the effects of 1/3 SW were compared with the separate effects of  $\text{Ca}^{2+}$  and  $\text{Na}^+$  ions. Fish were exposed to FW enriched with these ions at the same concentrations as in 1/3 SW.

In  $\text{Ca}^{2+}$ -enriched FW, the %PRL and the affinity of PRL cells for immunostaining are as high as in FW. Thus it can be concluded that the  $\text{Ca}^{2+}$ -concentration of 1/3 SW does not influence the development of PRL cells in *C. whitei*. The small decrease of the %PRL in  $\text{Ca}^{2+}$ -enriched FW can be attributed to the small increase of osmolality due to the addition of  $\text{CaCl}_2$ . A comparable decrease has been found in fish developing in water with an osmolality of 20 mOsm/kg. In these fish, after 3 weeks, the %PRL has declined to  $14.2 \pm 0.8\%$  (Ruijter et al. 1984).

Wendelaar Bonga and co-workers (1983) have observed that exposure of *S. mossambicus* to 2.5 and 5 mM  $\text{Ca}^{2+}$  reduces PRL-cell volume by 30 and 50%, respectively.

In *C. whitei* in  $\text{Na}^+$ -enriched FW, PRL-cell development is decreased to the same level as in 1/3 SW. No differences between  $\text{Na}^+$ -enriched FW and  $\text{Na}^+$ - and  $\text{Ca}^{2+}$ -enriched FW are observed (Fig. 7).

As mentioned in the introductory paragraph, the effects of environmental  $\text{Ca}^{2+}$  on PRL cells are different in several species of teleosts. *C. whitei* appears to be a species in which PRL cells are inhibited by increased osmolality or  $\text{Na}^+$  concentration but not by an increase in environmental  $\text{Ca}^{2+}$  concentration. In another cyprinodont, *F. heteroclitus*, PRL cells are only marginally affected by a  $\text{Ca}^{2+}$ -deficiency in SW; the PAS-positive cells of the PI (PAS cells) however are strongly activated (Ball et al. 1982). In contrast, PRL cells in *S. mossambicus* are strongly affected by changes in environmental  $\text{Ca}^{2+}$  (Wendelaar Bonga et al. 1983) but no changes are observed in PAS cells (Van Eijs and Wendelaar Bonga 1984). Similarly in *G. aculeatus* only PRL cells (Wendelaar Bonga 1978a, b) and not PAS cells (Wendelaar Bonga, unpublished observations) respond to changes in the ambient  $\text{Ca}^{2+}$  concentrations. *C. auratus* seems to take an intermediate position: in deionized water PAS cells, but not PRL cells, are inactivated when  $\text{CaCl}_2$  is added (Olivereau et al. 1980, 1982a); in 1/3 SW both cell types are slightly activated when  $\text{CaCl}_2$  is omitted (Olivereau et al. 1982b, 1983). These findings suggest that in a given species either PRL cells or PAS cells respond to changes in environmental  $\text{Ca}^{2+}$ . The functional significance of this phenomenon remains to be clarified.

The rate of development of PRL cells in young fish appears to be related in a similar way to environmental osmolality and  $\text{Ca}^{2+}$  concentration as the activity of PRL cells in adult fish. Therefore, we conclude that the analysis of the effects of environmental changes on PRL cell development in the early stages of growth of the fish can be used to determine PRL cell function.

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